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The Relation of Anatomy to Water Movement and Cellular Response in Young Barley Leaves[1]

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ABSTRACT

Young barley (*Hordeum vulgare* L. cv Arivat) leaves were examined anatomically and physiologically to infer the pathway of transpirational water movement and to explain why the growing region is more responsive to osmotic stress than the expanded blade. Vessels with open lumens extend from the intercalary meristem to the expanded blade, and all vessels are clustered in five vascular bundles that are separated by 20 closely packed mesophyll cells. Heat pulse transport data confirmed the anatomical suggestion that water moves through the growing region in vessels and not intercellularly, and also showed that stress reduces xylem water transport within 1 minute while transpiration remained unaffected. Water equal in volume to twice that expected in the xylem, and which exchanges more readily with water in the nutrient solution than with most water in tissues, can be extracted easily from growing tissues. It is hypothesized that this water is xylem plus cell wall water, that osmotic stress will quickly reduce its *in situ* water potential, and that stress causes growth to stop because cells in the growing region can respond rapidly to changes in water potential around them. In the expanded blade, bundles containing vessels are three and eight cells away from the closest and next substomatal cavities. This allows xylem water loss to occur predominantly through the closest stomata, and the expanded blade is believed to be less responsive because effects of stress on xylem water potential are confined largely to cells immediately around the vessels.

Elongation of many plant organs occurs largely in localized "growing regions," and recent studies have demonstrated that growing regions of unstressed barley (10), wheat (2), and corn (11) leaves, and also dark-grown soybean hypocotyls (3) can have significantly lower water potential values (ψ)[2] than expanded areas of the same organ. Additionally, experiments with intact young barley seedlings have shown that the growing region is highly responsive to water stress. As in corn (1), elongation of barley leaves stops almost immediately after roots are exposed to osmotic solutions (10). In barley seedlings stressed with mild to moderate concentrations of osmoticum (−0.2 to −1.0 MPa of PEG 8000), leaf expansion resumes in a few minutes to 1 to 2 h, with lag periods increasing and new growth rates decreasing with stress intensity. Such results indicate that most elongating cells, which are confined to the basal 1 cm of the leaf, rapidly sense and begin adjusting to changes in ψ of the root environment.

In addition to causing growth cessation, osmotic stress rapidly initiates other physiological alterations in the growing region. In stressed plants, significant and equal reductions in ψ and ψ_π are evident in 0.5 to 1 h, and increases in glucose account in part for the lowering of ψ_π (16). The adjustment normally continues for about 12 h before stabilization occurs, and in plants stressed with different concentrations of osmoticum, reductions in tissue ψ ultimately equal those applied in the nutrient solution (10, 16). Additionally, water stress reduces the percentages of ribosomes present as polyribosomes within 10 to 15 min (9); in plants stressed to different degrees, polyribosome percentages, after stabilization is achieved in 4 h, are correlated highly with growth rates. In contrast to the rapid and pronounced changes seen in growing tissues, stress-induced reductions in polyribosome percentages of expanded blades are not evident in 2 h (9) and decreases in ψ often are not significant even after 24 h (10, 16).

To explain why stress causes rapid growth cessation, it was hypothesized that reduced water availability causes an immediate reduction in the water potential of the transpiration stream, and cells in the growing region are able to respond to those changes (10). It was proposed also that the expanded blade was relatively insensitive to stress because water movement from the xylem to stomata largely bypassed most mesophyll cells. This view was supported by studies that showed that when [^{3}H]water was supplied to roots of intact plants, radioactivity first appeared as transpiration before it was detected in the blade (10). However, anatomical data suggesting the likely pathway of water movement in and from leaves were not available.

We have since performed extensive anatomical studies on various parts of the barley leaf and now present key structural and physiological data that clarify how water moves through growing and expanded tissues of barley leaves. Additionally, information from these and earlier studies was considered as a whole in order to outline how plant cells may respond to reduced water availability.

MATERIALS AND METHODS

Barley seeds (*Hordeum vulgare* L., cv Arivat) were obtained from Dr. R. T. Ramage, Research Geneticist, United States Department of Agriculture, Science and Education Administration, University of Arizona, Tucson. Seeds were germinated and grown at 25 ± 2°C with 13 h daily light (200 μmol m^{-2} s^{-1} PAR). After 4 d in vermiculite, roots were washed gently, and seedlings were transferred to racks and grown hydroponically overnight and studied on the 5th day.

For anatomical studies, 2- to 5-mm segments of leaf tissues were fixed in 3% (v/v) glutaraldehyde in 0.07 M, pH 6.8, sodium phosphate buffer for 1 to 2 d at 4°C, dehydrated, embedded in paraffin, sectioned to 12 μm, and stained in safranin-fast green according to Feder and O'Brien (7). Hand-cut cross-sections of fresh tissues were also prepared and measured with a micrometer for mesophyll areas and lumen areas of vessel elements. Sections

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[2] Abbreviations: ψ, water potential; ψ_π, osmotic potential.

were prepared on a slide and quickly immersed for 10 s in a drop of water containing 0.05% toluidine blue (15), then washed twice in near-isotonic solutions of sorbitol in water (−0.8 MPa for sections that were 1 and 5 mm from the seed, and −0.7, −0.6 and −0.5 MPa, respectively, for sections that were 15, 25, and 45 mm from the seed).

Heat pulse transport studies were performed to determine the likely pathway of water movement through the basal regions of intact, transpiring barley leaves. If water moves through lumens of vessels or the mesophyll as a whole, the velocity of transport of water labeled as a "warm spot" should equal calculated values based on their respective volumes. To perform studies with minimal disturbance to the plant, a Sensortek IT-23 microprobe thermocouple linked to a Sensortek BAT-12 digital thermometer (Sensortek, Inc., Clifton, NJ) was positioned in the fold of the first leaf 45 mm away from the point of attachment to the seed. Heat pulses were applied at different positions below the probe and were generated by applying current for 0.2 s from a 16 V, 2A DC power supply (Hampden Model BPS-16-P, Hampden Engineering, E. Longmeadow, MA) across a 100-mm length of 0.3 mm diameter stainless steel wire shaped to partially surround the leaf and held 2 to 3 mm away. This 0.2-s pulse causes no damage and effects an instantaneous 10°C increase in tissue temperature at the source, and a temperature elevation of about 1.5°C is ultimately detected 10 or more mm away. In heat pulse transport velocity experiments, the heat source was held 20 or more mm away from the probe, and a 0.1°C rise in temperature was used to monitor velocities. Temperature fluctuations around the probe were minimized by loosely wrapping the folded area of the leaf with insulation, and transpiration was regulated by adjusting light intensities. In some cases, transpiration rates were reduced by adding solutions containing either NaCl or PEG, which gave identical results in the short time studies performed here. Transpiration rates were estimated by gravimetrically measuring water loss from groups of 6 to 10 plants simultaneously grown under conditions used in the heat pulse studies. The basal regions of leaves of plants used in transpiration measurements were held with polyurethane foam and placed over foil-covered, 50-ml plastic beakers containing nutrient solutions. In studies designed to measure rapid effects of osmotic stress on transpiration, the plants were transferred to fresh nutrient solutions with or without osmoticum and weighed immediately after transfer and also at 5-min intervals.

Studies with ^{3}H-labeled water were performed to infer how water moves in its passage from the roots through the leaves. To reduce the possibility of contamination of leaf tissues, the lower half only of roots of 10 intact plants was exposed to nutrient solutions containing ^{3}H-labeled water of about 15,000 dpm/μl specific radioactivity. Collection and handling of transpired water, water from whole leaf tissues, and water remaining in tissues following extraction of a small amount by centrifugation were performed as previously noted (10). Roots were blotted with tissue paper, and water from the sections exposed to labeled solution was also extracted and counted.

Because grasses have parallel veins, it was reasoned that water in vessels but not in membrane-bound cells could be extracted easily by centrifuging fresh-cut sections against an adsorptive surface. The centrifugation procedure employed to extract water from tissues was modified from a procedure used to obtain cell wall proteins (12). Groups of 10 sections that were 10 mm in length were inverted and placed into tared 1.5-ml, cappable polypropylene centrifuge tubes that contained four discs of white tissue paper cut to 8 mm in diameter and held in previously formed paraffin film cups. Inversion of tissues ensured that sections of the growing region had uniform fresh-cut ends that were in contact with the tissue papers. Tubes were centrifuged for 1 min at 5g (250 rpm in a Sorval Type M rotor and GLC centrifuge), leaf segments were then removed, the tube was quickly reweighed, and discs were then transferred into scintillation vials for counting. Because amounts of water were small, extreme care was taken to work quickly while avoiding weighing errors.

RESULTS AND DISCUSSION

Anatomical Studies of Growing and Expanded Regions of Barley Leaf Blades. Although extensive studies were performed on various sections of the young barley leaf, the anatomical features found in the section from the intercalary meristem (Fig. 1A) and the midblade region (Fig. 1B) provide a clear basis for suggesting how transpirational water will move throughout the leaf.

Vascular bundles with mature as well as immature vessel elements are present in the intercalary meristem (Fig. 1A) as well as in the expanded blade (Fig. 1B) of young barley leaves. The occurrence of mature vessel elements that interconnect end to end to form vessels that extend through the meristem and elongating region of barley is analogous to the anatomy found in corn (5). Since the mesophyll cells in the growing region are closely packed in fresh-cut (data not shown) as well as in fixed sections (Fig. 1A), and since immature vessel elements have membranes that prevent mass water transport (13), transpirational water should move through the growing region of grass leaves via the large diameter lumens of vessels as suggested by Westgate and Boyer (19) rather than intercellularly as we (10) previously believed.

All vessels are clustered in only five vascular bundles throughout the leaf of 5-day-old barley seedlings (Table I), and these bundles are separated by 20 closely packed mesophyll cells and one to three immature vascular bundles (Fig. 1, A and B). In the expanded blade, the relationships of stomata and mesophyll cells to the five vascular bundles with vessels provide an anatomical basis for explaining why labeled water given to roots appears as transpiration before substantial labeling of the blade occurs (10; also, Fig. 4, bottom right curves). As can be inferred by examining the 12 μm thick section of the expanded blade shown in Figure 1B, stomata are regularly distributed on both the adaxial and abaxial surfaces of the expanded blade and are associated closely with immature bundles as well as with the five bundles that transport water. Since bundles with functional vessels are only three cells away from the closest, and eight or nine cells away from the next substomatal cavity, water movement out of the leaf will occur predominantly from the closest stomata. Because the specific radioactivity in the blade becomes equal to the transpired water after 3.5 h, a more limited movement of water from the xylem to more distal stomata also occurs.

Heat Pulse Transport Studies. Heat pulse transport velocity studies have been used to relate transpiration rates to water transport in herbaceous and woody plants, and theoretical and practical aspects have been discussed elsewhere (17, 18). In order to develop a suitable method for measuring heat pulse transport rates through the basal area of barley leaves, it was assumed that transpirational water movement through already expanded tissues will occur via the lumen of vessels only. Accordingly, the thermocouple probe was held in the fold of the leaf 45 mm from the seed, and heat pulses were applied to expanded tissues 20 or 30 mm away from the probe. Data from plants transpiring at three different rates show that observed times required to reach the probe from these points are identical to calculated values (Table II) based on mean vessel lumen volumes derived from data in Table I. When the procedure was extended to the growing region of the leaf, where it had been suggested earlier that transpirational water moved intercellulary (10), the time periods required to go the 45 mm from the seed to the probe were again found to equal values calculated for movement through vessels

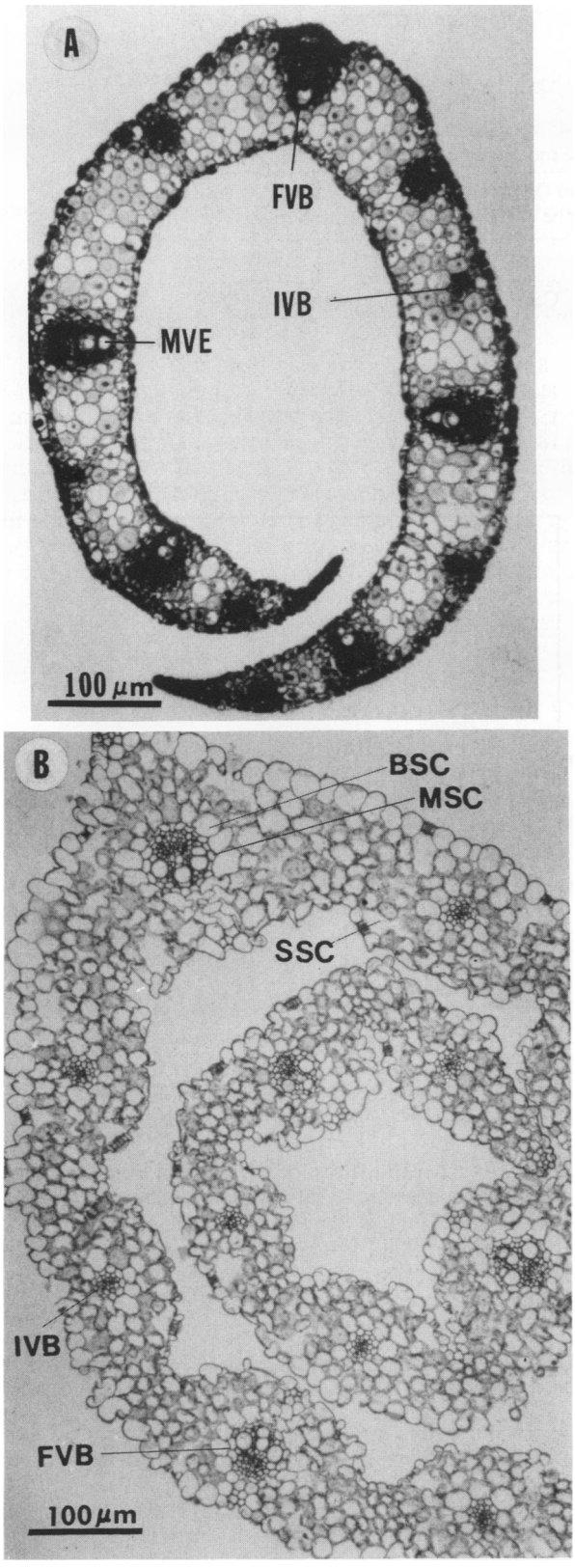

FIG. 1. Micrographs from barley leaves. A, Cross-section at the intercalary meristem showing mature vessel elements (MVE) in functional vascular bundles (FVB) and immature vascular bundles (IVB) with no MVE. B, Expanded blade showing association of substomatal cavity (SSC) with FVB and IVB. FVB and IVB are surrounded by thin-walled bundle sheath cells (BSC) and mestome sheath cells (MSC). Cross-sections of the intercalary meristem and expanded blade were obtained 1 and 70 mm from the seed, respectively.

Table I. *Mesophyll and Vessel Areas and Numbers of Functional and Immature Vascular Bundles in Different Areas of Young Barley Leaves*

Values (±SD) are means from five or more fresh-cut cross-sections.

Distance from Seed	Mesophyll Area	Vessel Area	Numbers of Vascular Bundles	
			Functional	Immature
mm	*mm²*	*mm²*		
1	0.40 ± 0.013	0.0029 ± 0.0002	5.5 ± 0.5	3.5 ± 0.5
5	0.43 ± 0.010	0.0036 ± 0.0004	5.2 ± 0.4	8.3 ± 0.7
15	0.55 ± 0.029	0.0041 ± 0.0001	5.1 ± 0.5	8.6 ± 0.5
25	0.76 ± 0.017	0.0044 ± 0.0002	5.1 ± 0.3	9.5 ± 0.7
45	0.98 ± 0.027	0.0048 ± 0.0003	5.0 ± 0.0	10.7 ± 0.7

alone (Table II). In another approach, the thermocouple probe was held 45 mm from the seed, and differences in times required to go 45 and 35 mm on the same seedlings were then determined. Using the high, medium, and low transpiration conditions of Table II, the calculated times required to move through the basal 10 mm (vessel volume = 0.034 mm^3) were 1.2, 2.2, and 4.1 s, whereas the observed values were 1.5 ± 0.2; 2.5 ± 0.4, and 4.6 ± 0.6 s. When the heat pulse data are considered together with the anatomical data for corn (5) and for barley (Fig. 1A) and also with studies of Barlow (2), who found that dyes provided through cut roots of wheat appear in only a few distinct regions in the growing areas of their leaves, it seems reasonable to conclude that movement of water through the intercalary meristem and expanding zones of cereal leaves in general will be primarily through the xylem.

If osmotic stress reduces water entry and effects the predicted (10) rapid reduction in xylem ψ, it should also cause xylem water transport to drop rapidly. This possibility was supported by heat pulse transport studies (Fig. 2), which showed that transport velocities from 25 to 45 mm away from the seed were reduced within 1 min following exposure to NaCl solutions of −0.8 MPa. This reduction in xylem water transport is not due to reduced transpiration because water loss was not affected in similarly treated plants for at least 5 min (Fig. 3). The transpiration data agree with results of others (6, 8) who have shown that stress does not cause an immediate closure of stomata. In addition to supporting the view that osmotic stress will reduce xylem ψ, the heat pulse data considered together with the transpiration results indicate that other factors, such as ABA transported in the xylem of stressed plants (4), may be responsible for stomatal closure.

Transport and Exchange of Labeled Water in Barley Seedlings. In a further effort to trace water movement through leaves, [^{3}H]water was supplied to roots of intact seedlings, and changes in specific radioactivities of water in different tissues were measured over a time course (Fig. 4).

Roots of young barley seedlings that are immersed in labeled nutrient solution rapidly reach and maintain a specific radioactivity about 0.9 times that of the nutrient solution. In other, shorter duration studies, specific radioactivities of water in immersed and blotted roots were found to be 0.3, 0.7, and 0.9 times that of the nutrient solution after 10 s and 5 and 15 min, respectively. Such data show that radioactivities measured for water in roots are not due simply to surface adherance and that most but not all of the water in roots exchanges readily with water in the nutrient solution.

It was hypothesized originally that centrifugation of water from sections of the growing region of leaves will extract only xylem water. However, data from many studies show that 10 sections from the basal 10 mm of the leaf yield an average of 0.7 mg of water following 5 × g centrifugation (0.8 mg when centrifuged at 15 × g), whereas an estimate based on total tissue water content (46 mg) and mean xylem/mesophyll volume ratios (1/

Table II. *Calculated and Observed Times Required for Water to Move through Different Lengths of Barley Leaf Xylem*

High, medium, and low transpiration rates (0.0225; 0.0117; 0.0056 mm^3 water $plant^{-1}s^{-1}$, respectively) were obtained with 350 μmol m^{-2} s^{-1} light at 28°C; 200 μmol m^{-2} s^{-1} at 25°C; 200 μmol m^{-2} s^{-1}, 25°C and −0.8 MPa NaCl, respectively. The thermocouple probe was held 45 mm from the seed, and heat was applied at indicated distances from the probe. Observed values (±SD) are means of eight determinations.

Distance to Probe	Total Vessel Lumen Volume	Calculated (Calc) and Observed (Obs) Times Needed for Water to Reach Probe at Indicated Transpiration Rate					
		High		Medium		Low	
		Calc	Obs	Calc	Obs	Calc	Obs
mm	mm^3			*s*			
20	0.094	4.2	4.1 ± 0.5	8.1	7.8 ± 1.2	16.8	16.4 ± 1.4
30	0.14	6.2	5.9 ± 0.5	11.9	12.2 ± 1.2	24.7	23.7 ± 1.6
40	0.18	7.9	7.7 ± 0.6	15.3	16.8 ± 1.4	31.8	30.8 ± 1.8
45	0.20	8.7	9.5 ± 0.8	16.9	19.2 ± 2.3	35.1	33.7 ± 1.6

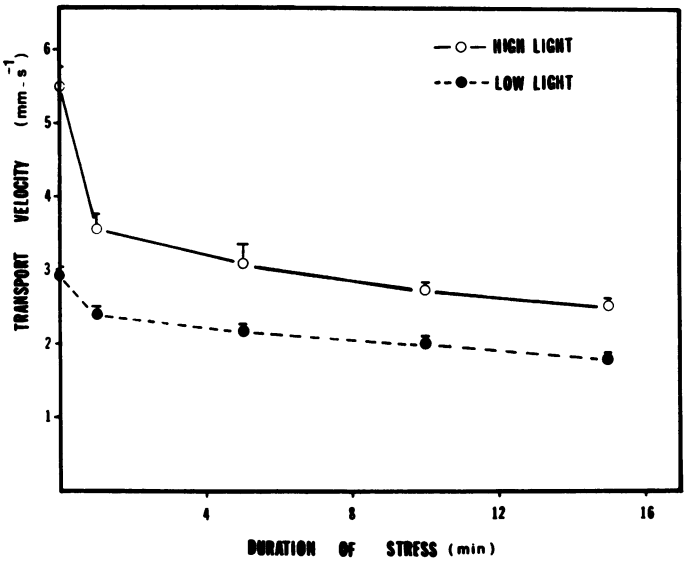

FIG. 2. Effect of sudden osmotic stress on heat pulse transport velocities. Unstressed plants were maintained for 2 or more h under high (400 μmol m^{-2} s^{-1}) or low (200 μmol m^{-2} s^{-1}) light, then stressed with −0.8 MPa solution of NaCl. Heat pulse transport velocities measured between 25 and 45 mm from the seeds were determined prior to or at indicated times after stress, and values (±SD) are means of five observations.

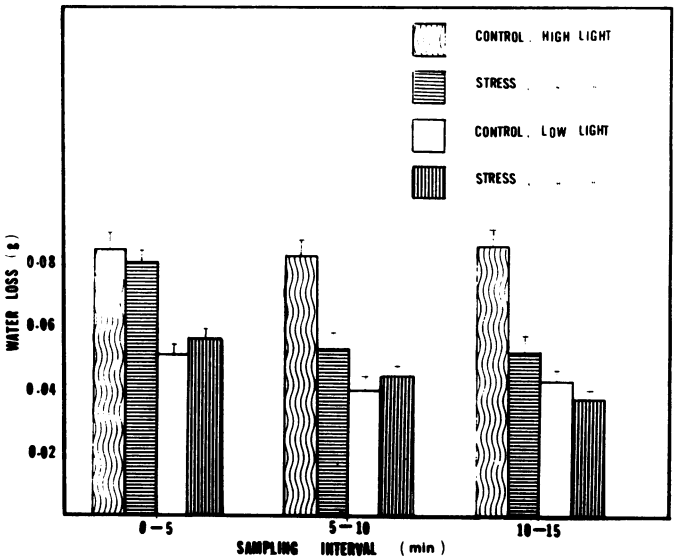

FIG. 3. Osmotic stress effects on transpiration. Transpirational water losses in 5-min intervals were determined under conditions used in Figure 2, and values (±SD) are means of nine observations.

120, derived from data in Table I) suggests there should be 0.38 mg of water in the lumen of vessels.

When the growing region of leaves was sampled 2.5 h after roots of intact seedlings were exposed to [^{3}H]water, the specific radioactivities of centrifuged and residual waters were 0.8 and 0.5 times that of the nutrient solution; after 24 h, the respective values were 1.0 and 0.7 times that of the medium. Although confirming studies must be performed, we suggest that the water obtained by centrifuging cut sections of the growing region is apoplastic water (xylem water plus probably cell wall water) because it is easily extractable, because its volume is about twice that calculated to be present in the xylem, and because it equilibrates more readily with the nutrient solution than with much of the water in the rest of the growing tissue. The water obtained by centrifugation of the leaf section 40 to 50 mm from the seed showed similar changes in radioactivities, but the increase in labeling was delayed. The results are the type expected if water brought into tissues via the xylem exchanges relatively easily with endogenous unlabeled water in the xylem plus a small amount of additional apoplastic water, but exchanges with more difficulty with water in the rest of the leaf. Additionally, because the specific radioactivity of the extracted water obtained after 24-h labeling is equal to that of the nutrient solution and is clearly higher than that of the residual water, contamination by water from broken cells at the cut ends of sections of the growing region of leaves is minimal.

How Might Barley Leaf Cells Sense and Respond to Water Deficits?

In brief summary, the heat pulse transport data (Table II) confirm the anatomical suggestion (Fig. 1A) that transpirational water should move in vessels through the basal region of barley leaves. Since sudden osmotic stress will reduce xylem water transport rates within 1 min (Fig. 2), while not affecting transpiration for at least 5 min (Fig. 3), there is now additional support for the idea (10) that sudden reduced availability of water to roots together with ongoing transpiration will reduce the hydrostatic pressure and, therefore, the ψ of the xylem in leaves. Because stress causes almost immediate cessation of barley leaf elongation (10) and rapid initiation of osmotic adjustment (10, 16) and reductions in the percentages of ribosomes present as polyribosomes (9) in the growing region, effects of stress on the

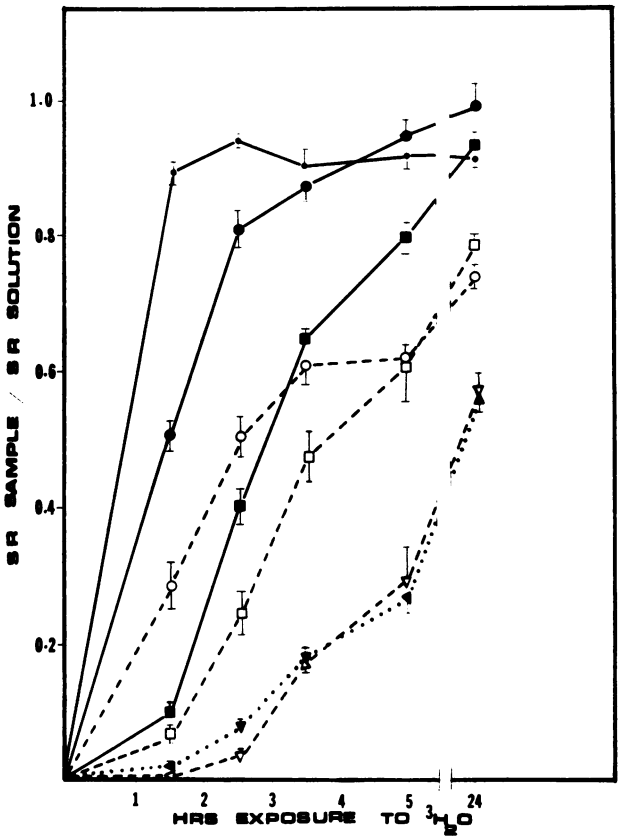

FIG. 4. Time course changes in specific radioactivities (SR) of water in different parts of young barley seedlings. ^3H_2O was supplied to the lower half of roots and at intervals were measured SR of water in roots (•), the upper blade 60 to 110 mm from the seed (△), and transpired water (▲). SR of water extracted by centrifugation (CW) (●) and residual water (○) from the growing region 0 to 10 mm from the seed and CW (■) and residual water (□) in the expanded lower blade 40 to 50 mm were also obtained. All values are expressed as a fraction of SR found in the nutrient solution and represent the mean (±SD) of three determinations.

xylem's ψ must be transferred laterally from vessels in five widely separated bundles to all closely packed intervening cells (Fig. 1A).

Although our views may be modified as more data become available, we suggest that stress-caused reductions in xylem ψ are transferred through pits of vessels and into the walls of the mesophyll cells in the growing region and that the plasma membrane is the major barrier for water movement. Further, we infer that rapid and prolonged stress effects on growth and the physiology of growing tissues will occur because mesophyll cells can sense and respond to alterations in the ψ of the apoplasm around them, and not because they first change in turgor.

The water extracted easily by centrifugation, which represents about 2% of the total water in growing regions, has some of the properties expected for apoplastic water. We are now examining the relationships existing between the pits in vessels and the walls of mesophyll cells, and we are also further characterizing the water obtained by centrifugation against an adsorptive surface.

Although there is no direct evidence that shows the plasma membrane is a major barrier for water movement, this possibility can be inferred for several reasons. The labeling data showing that the easily extracted water has a higher specific radioactivity than the remaining 98% of the water in tissues (Fig. 4) make it clear that water does not move freely throughout leaf tissues. The heat pulse transport studies (Fig. 2) support the view that xylem hydrostatic pressure is reduced rapidly by stress. Reductions in xylem hydrostatic pressure without a detectable reduction in tissue water status can occur only if water movement from the xylem to the mesophyll cells is restricted and if water in the apoplasm is a small fraction of the total water present in leaves. Several groups (2, 3, 10, 11) have shown that growing regions can have lower ψ than those of expanded tissues, which may only be a few millimeters away. Nonami and Boyer (14) feel that growing regions have low ψ because water entry from the xylem into expanding cells lags behind turgor reductions caused by elongation. Finally, osmotic adjustment with constant turgor maintenance, as seen in growing regions of leaves of stressed barley seedlings (9, 10, 16) and in many other tissues, can occur because net water entry into cells is somehow coordinated with solute accumulation.

Because turgor maintenance appears to be a high priority process in stressed plant tissues, it becomes increasingly difficult to believe that growth in many tissues should be controlled by turgor. Furthermore, anatomical and physiological considerations show that the water status of mesophyll cells in intact tissues can be altered only via changes in the water status of the xylem. Although the mechanisms have not been defined, it is possible to imagine how mesophyll cells, which are at the end of the logistical pipeline from the xylem, can respond to changes in water status of the xylem. In contrast, it is very difficult to suggest how changes in the turgidity of mesophyll cells can rapidly reduce the proportion of ribosomes present as polyribosomes or lead to osmotic adjustment.

Cells in the expanded blade may also respond to changes in the surrounding apoplastic ψ, and the available evidence indicates that their low sensitivity to stress occurs because most mesophyll cells are largely uncoupled from stress effects on the xylem. Transpirational water loss coupled with restricted water availability is the likely basis for reduced xylem hydrostatic pressures. From an anatomical standpoint, views such as those in Figure 1B show that water is brought into the blade in only five widely spaced vascular bundles, and they suggest also that water should be lost from the stomata that are closest to the bundles. This suggestion is supported by labeling studies that show that [^{3}H] water given to roots appears as transpired water before the blade becomes heavily labeled (Fig. 4). Probably because of relatively high resistances to water movement across or through the mesophyll cells in the blade, the water status of cells immediately around the xylem may differ substantially from that of those in much of the rest of the mesophyll.

The data and discussion presented here provide reasons for believing that leaf anatomy plays a major role in determining how cells respond to water stress. Because leaves vary considerably in structure and also in their response to stress, it should also be possible to clarify further how anatomy restricts water movement and how it affects cellular responses to osmotic stress.

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